

Preparation of Protein-containing Extracts from Microbiota-rich Intestinal Contents

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[Abstract] The contribution of microbiota in regulating multiple physiological and pathological host responses has been studied intensively in recent years. Evidence suggests that commensal microbiota can directly modulate different populations of cells of the immune system (*e.g.*, Ivanov *et al.*, 2008; Atarashi *et al.*, 2011). Recently, we showed that protein extracts from gut commensal microbiota can activate retina-specific T cells, allowing these autoreactive T cells to then break through the blood-retinal barrier and trigger autoimmune uveitis in the recipient (Horai *et al.*, 2015). The protocol below describes the method to prepare intestinal protein-rich extracts that can be used in various *in vitro* and *in vivo* immunological studies.

[Background] Intestinal microbiota represent a complex community of microbes that provide a wide variety of innate and adaptive stimulants. Their isolation and purification from stool samples has been performed and protocols have been published (Mueller and Pan, 2013; Verberkmoes *et al.*, 2009; Tanca *et al.*, 2014; Xiong *et al.*, 2015a; Xiong *et al.*, 2015b). Most of these protocols have been developed with the aim of performing proteomic studies for characterization of the microbiota. Consequently, although they emphasize protein yield and purity, they are time consuming and may include a protein denaturing step that affects protein structure (Verberkmoes *et al.*, 2009) or use reagents (*i.e.*, sodium azide, SDS, phenol) that are incompatible with subsequent cell culture based assays (Tanca *et al.*, 2014; Xiong *et al.*, 2015a; Xiong *et al.*, 2015b). These characteristics are not desired when functional immunological assays are intended to be performed with the extracted proteins.

We have developed a simple and fast method that can be used to obtain protein-rich extracts from different areas of the intestine as well as from stool samples. The protocol does not include denaturing steps and the protein-rich extracts can be used in different *in vitro* and *in vivo* immunological assays with live cells, including T cell stimulation for proliferation and for adoptive transfer (see Data analysis section).

Materials and Reagents

1. 50 ml centrifuge tubes (Corning, Falcon®, catalog number: 352070)
2. 15 ml centrifuge tubes (Corning, Falcon®, catalog number: 352095)
3. 100 mm culture dish (Corning, Falcon®, catalog number: 353003)
4. Sterile 12 ml syringe (COVIDIEN, Monoject™, catalog number: 8881512878)
5. 23 G x 1" needle (COVIDIEN, Monoject™, catalog number: 8881200383)
6. 3 ml sterile syringe (COVIDIEN, Monoject™, catalog number: 8881513934)
7. Sterile 1 ml syringe (COVIDIEN, Monoject™, catalog number: catalog number: 8881501400)
8. 1.5 ml microtubes (Eppendorf, catalog number: 022-36-411-1)
9. Syringe filter 0.22 µm, polyethersulfone, 33 mm, gamma sterilized (EMD Millipore, catalog number: SLGV033RS)
10. Sterile 1x PBS (Thermo Fisher Scientific, Gibco™, catalog number: 10010-023)
11. Pierce™ Coomassie plus Bradford protein assay kit (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 23236)
12. 10 mg/ml aprotinin (Sigma-Aldrich, catalog number: A1153)
13. 10 mg/ml leupeptin (Sigma-Aldrich, catalog number: L9783)
14. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
15. Protease inhibitor cocktail (see Recipes)

Equipment

1. CO₂ mouse euthanasia chamber (with flow meter) (Euthanex, model: E-20028)
2. -80 °C freezer (Thermo Fisher Scientific, Thermo Scientific™, model: Revco UXF)
3. Analytical balance (Mettler-Toledo International, model: AE50)
4. Refrigerated centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Legend XFR)
5. Refrigerated microcentrifuge (Eppendorf, model: 5424R)
6. Vortex-Genie 2
7. Sonicator (Heat systems, model: XL2010)

Procedure

The following procedure describes the steps to collect the contents from the large intestine of one mouse. The protocol can be adapted to the collection of small intestine contents as well.

1. Euthanize mouse according to institutional guidelines using a CO₂ euthanasia chamber.
2. Cut the skin of the abdomen without disrupting the peritoneum (Figure 1A).
3. Cut the peritoneum to expose the small and large intestines (Figure 1B).
4. Carefully push the intestines out of the abdomen and isolate the complete small and large intestines by cutting just after the stomach and as low as possible of the colon (Figure 1C).
5. Remove large intestine from the beginning of the cecum to the end of the colon. Transfer intestine to a 100 mm culture dish placed on ice (Figure 1D).
6. Flush out intestinal contents with up to 10 ml of ice-cold PBS using a 12 ml syringe + 23 G x 1" needle (Figure 1E, F).
7. Disperse intestinal contents in the PBS by applying pressure with a 3 ml syringe plunger (Figure 1G). Transfer contents to a 50 ml tube (Figure 1H).
8. Pellet the contents by centrifugation at 2,000 x *g* for 10 min at 4 °C (Figure 1I).
9. Pour off and discard the supernatant by gentle inversion of the tube.

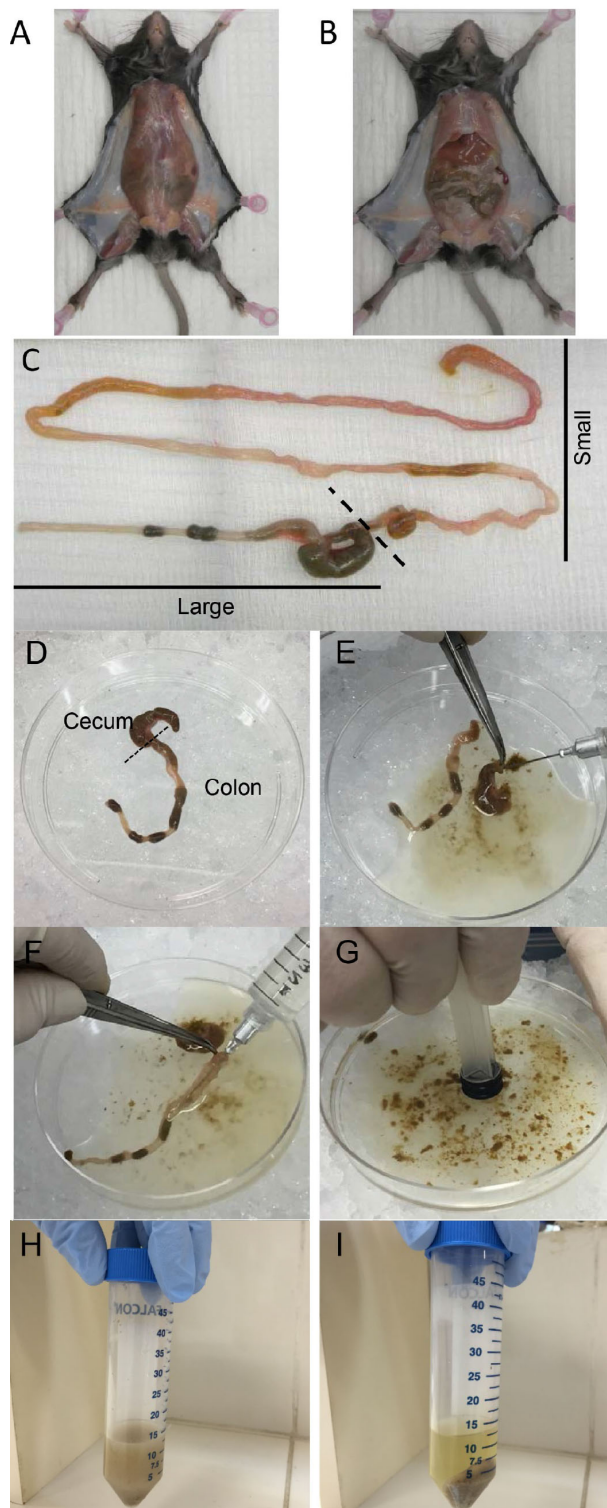


Figure 1. Intestinal contents collection. A. Opening the skin of the abdomen without disrupting the peritoneum. B. Opening peritoneum to expose intestine. C. Isolation of complete small and large intestine. D. Large intestine. E and F. Flushing out cecum (E) and colon (F) contents with PBS. G. Dispersing intestinal contents by using a plunger. H. Transfer contents into a 50 ml tube. I. Pellet contents after centrifugation.

10. Tare analytical balance with empty 50 ml tube and adjust to zero. Weigh the tube with pelleted intestinal contents.
11. Resuspend the pellet in freshly prepared protease inhibitor cocktail (see Recipes) at a concentration of 2 g/ml.
12. Freeze pellet in -80 °C freezer for 20 min, then thaw for 20 min and vortex at maximum speed for 5 sec to disrupt bacterial cells. Repeat the freeze/thaw/vortex cycle two additional times.
13. To complete cell lysis, sonicate the contents for 30 sec on ice, followed by 30 sec rest on ice. Sonicate 5 times in total (use power setting #2 for up to 2 ml of sample with fixed vibration frequency at 20 KHz) (Figure 2).

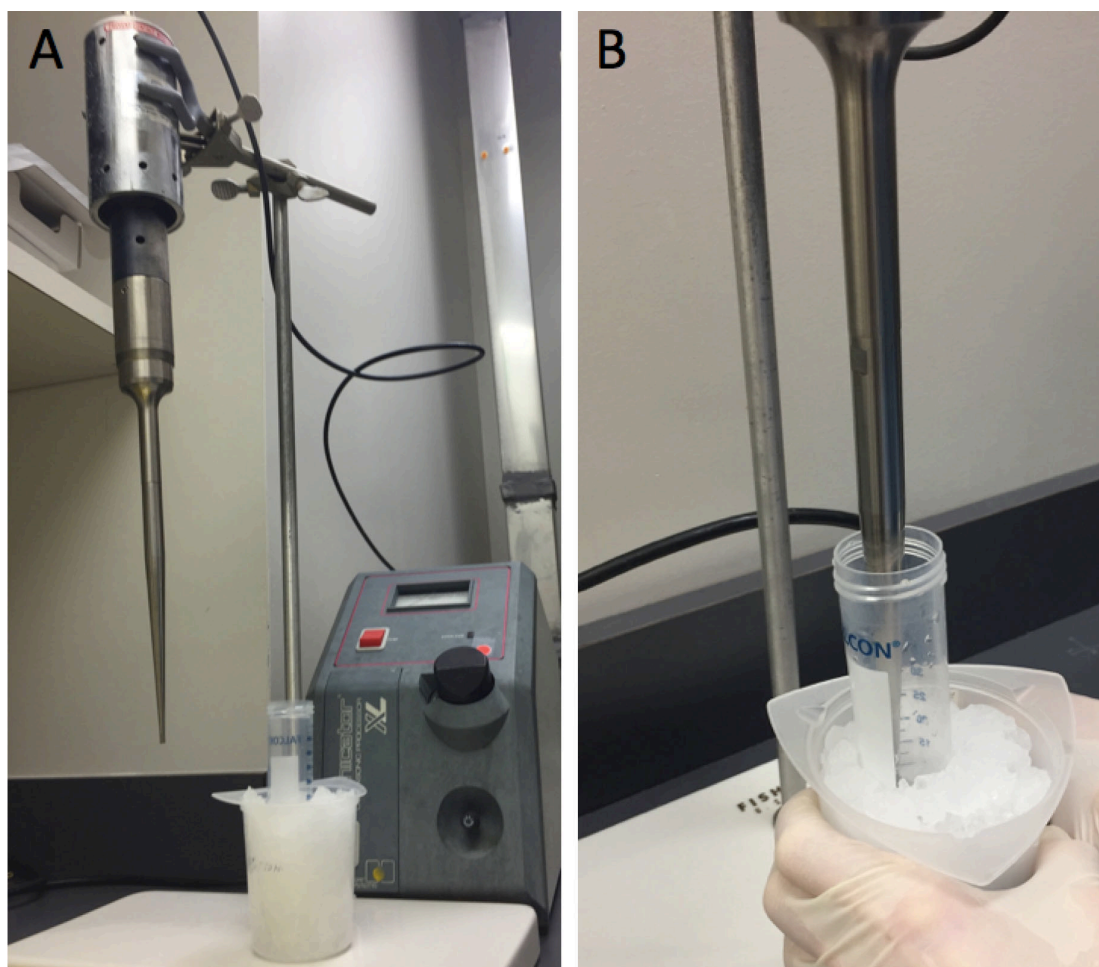


Figure 2. Cell lysis of the intestinal contents by sonication. Following three freeze/thaw/vortex cycles, contents are sonicated on ice. The sonicator apparatus (A) and the procedure (B).

14. Transfer the contents to 1.5 ml microtubes and centrifuge at 14,000 x *g* for 30 min at 4 °C.
15. The protein-rich supernatant is aspirated (typically 1 to 2 ml) without disturbing the pellet and collected into a 15 ml tube placed on ice.
16. Sterilize the extract using a 1 ml syringe and a 0.22 µm syringe filter (Figure 3).

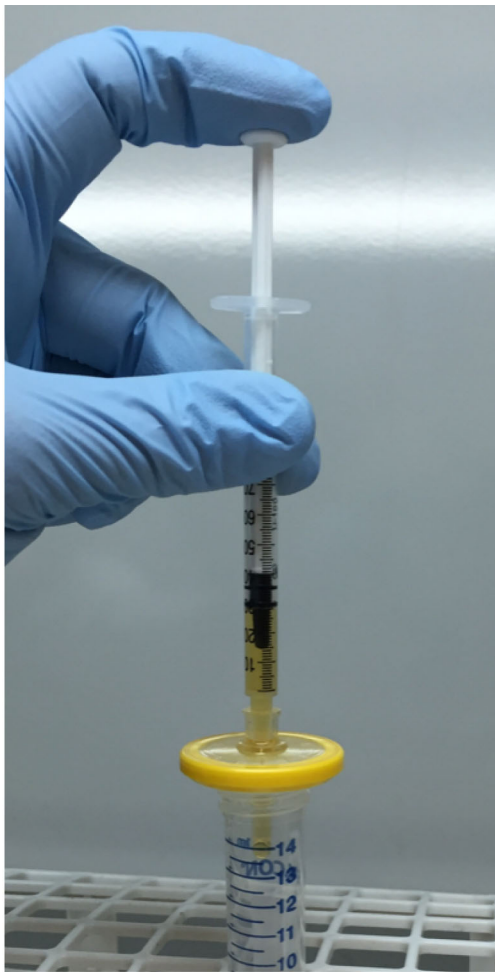


Figure 3. Sterilization of the extract. After centrifugation, bacteria-rich protein supernatant/extract is collected and passed through a 0.22 μ m syringe filter.

17. Determine protein concentration of extract using Bradford protein assay kit or equivalent, following manufacturer's protocol. We suggest a neat, 1:10, 1:100, and 1:1,000 dilution of the extract for the assay to ensure that the result falls on the standard curve.
18. The amount of extract to be used to stimulate cells *in vitro* may vary according to the experimental design. In the original article (Horai *et al.*, 2015), 10-500 μ g/ml of extract was used to stimulate retina-specific autoreactive T cells *in vitro*.

Data analysis

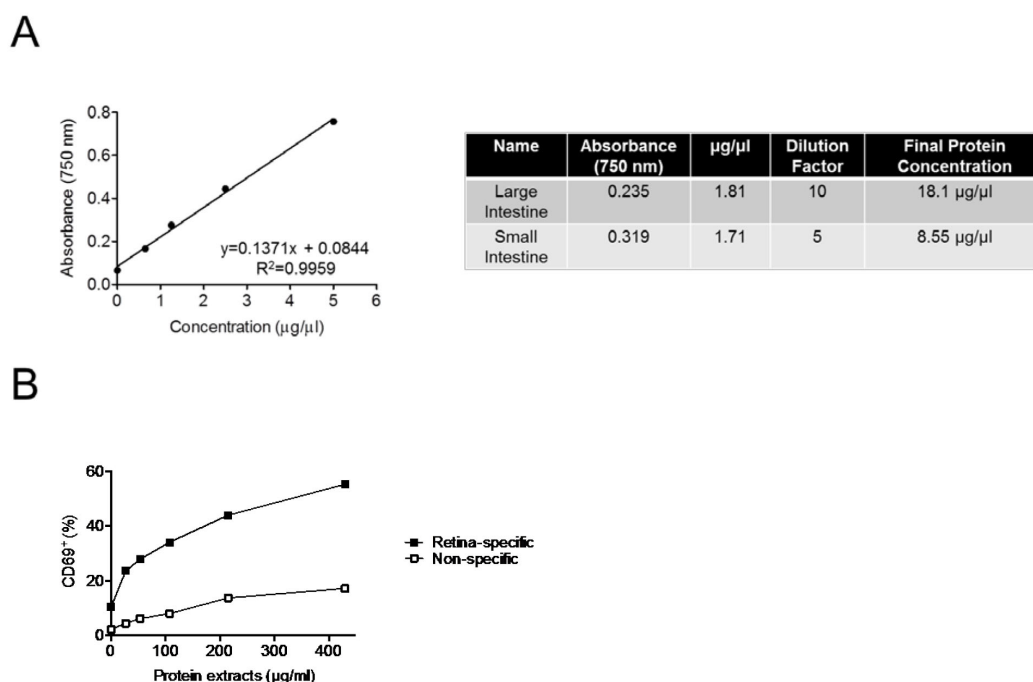


Figure 4. Representative results. A. Standard curve generated from protein assay kit (left) and typical final protein concentrations of bacteria-rich protein extracts obtained from large and small intestines (right). B. Induction of CD69 expression in retina-specific versus non-specific T cells from R161H mice after 20 h of stimulation with the extracts (adapted from Figure 6C, Horai *et al.*, 2015).

Recipes

1. Protease inhibitor cocktail
PBS supplemented with:
10 µg/ml aprotinin
10 µg/ml leupeptin
0.5 mM phenylmethylsulfonyl fluoride (PMSF)

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